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Calmodulin Lobotomized: Novel Insights into Calcium Regulation of Voltage-Gated Calcium Channels

Calcium entry via voltage-gated calcium channels can be regulated by calcium- and voltage-dependent inactivation mechanisms. For years, only L-type channels were thought to be capable of calcium-dependent inactivation (CDI). In this issue of *Neuron*, Liang et al. present evidence to show that CDI is a much more widely applicable feedback mechanism that limits calcium entry in response to rising intracellular calcium.

The entry of calcium ions via voltage-gated calcium channels triggers a number of cellular responses, including the release of neurotransmitters and the initiation of gene transcription. However, excessive calcium influx is toxic to cells, and as a consequence, calcium channels have evolved intrinsic inactivation mechanisms to limit the amount of calcium entry. In non-L-type calcium channels, inactivation has been thought to occur virtually exclusively through a voltage-dependent mechanism, that is, the channels cease activity in response to prolonged membrane depolarization. The channel structural determinants that underlie voltage-dependent inactivation (VDI) have been examined at length, and based on chimeric and mutagenesis studies, the pore-lining S6 transmembrane segments as well as several cytoplasmic regions of the calcium channel α_1 subunits have been implicated in the VDI process (Stotz et al., 2003). Based on these collective findings, it has been suggested that VDI may occur through a classical hinged lid mechanism, with the intracellular domain I-II linker region acting as a physical inactivation gate that prevents current flow by docking to the S6 segments. Yet, other intracellular regions, such as the amino and carboxyl termini, as well as the domain III-IV linker appear to be important for regulating VDI rates, perhaps by directly or allosterically interacting with the inactivation machinery (for review, see Stotz et al., 2003).

Although L-type calcium channels also display some degree of VDI, their overall inactivation profile is dominated by a calcium-dependent process, such that they display only weak inactivation in barium saline but robust inactivation in calcium-containing external solutions. Indeed, this regulation serves as an essential feedback mechanism that allows excitable cells to terminate calcium entry in response to rising intracellular calcium concentrations. It is now widely accepted that calcium-dependent inactivation (CDI) is critically dependent on the association of calmodulin with an IQ motif in the carboxyl terminus of the L-type calcium channel α_1 subunit (Peterson et al., 1999; Zuhlke et al., 1999; Erickson et al., 2003) and an ensuing transduction of the CDI process by a calcium binding EF hand motif located upstream of the IQ sequence (Peterson et al., 2000). Hence, although CDI and VDI appear to be functionally distinct mechanisms, there appears to be at least a partial overlap in the underlying channel structural determinants.

The widely held belief that only L-type calcium channels display CDI was first challenged by Lee et al. (1999), who demonstrated calmodulin-mediated CDI of transiently expressed P/Q-type calcium channels. Like the L-type, the P/Q-type channel contains an EF hand and an IQ-like motif in the C-terminal region, thus indicating that the basic principle underlying CDI is conserved in these two channel types (de Maria et al., 2001). Although both motifs are also present in the C termini of the N-type and R-type calcium channel α_1 subunits, CDI has not typically been observed with these calcium channel subtypes. An exciting study by Liang et al. (2003 [this issue of *Neuron*]), however, challenges this notion and provides novel insights into calcium/calmodulin regulation across the entire high-voltage-activated calcium channel family.

Using in situ FRET assays, Liang and coworkers elegantly demonstrate that calmodulin is capable of physically associating with both N-type and R-type channels. The functional implication of this association becomes apparent when the standard 5 mM EGTA concentration in the patch pipette is reduced to 0.5 mM, such that the calcium buffering capacity of the internal recording solution more closely reflects that of intact cytoplasm. In doing so, Liang and colleagues are able to unmask CDI in both N-type and R-type calcium channels, revealing dramatically accelerated inactivation kinetics of these channels upon switching from barium- to calcium-containing external solutions. Under these circumstances, the magnitude of CDI appears to be comparable to that observed with L-type calcium channels. This suggests that, at least at membrane potentials where significant calcium influx occurs, CDI may be the predominant inactivation mechanism in most types of high-voltage-activated calcium channels under physiological conditions. In analogy to what was just recently shown for L-type calcium channels (Erickson et al., 2003), the presence of calmodulin also appears to affect the rate of VDI in N-type and R-type channels. Carboxy-terminal mutant forms of these two channel types that display a reduced affinity for calmodulin show accelerated VDI kinetics, and overexpression of calmodulin is able to restore the slowly inactivating phenotype in both channels by mass action. Hence, it is reasonable to conclude that the binding of calmodulin to the L-type, N-type, and/or R-type calcium channel C termini antagonizes VDI. This is consistent with previous studies implicating the C-terminal regions of L-type (Soldatov et al., 1998; S.C. Stotz and G.W.Z., 2002, Soc. Neurosci., abstract) and P/Q-type channels (Sandoz et al., 2001) in regulating VDI; however, the precise molecular mechanisms by which the C-terminal/calmodulin complex interacts with other regions known to be critical for VDI (see above) remain to be determined. The intriguing implication of these findings, however, is that calmodulin interactions with the C terminus regions of L-type, R-type, and

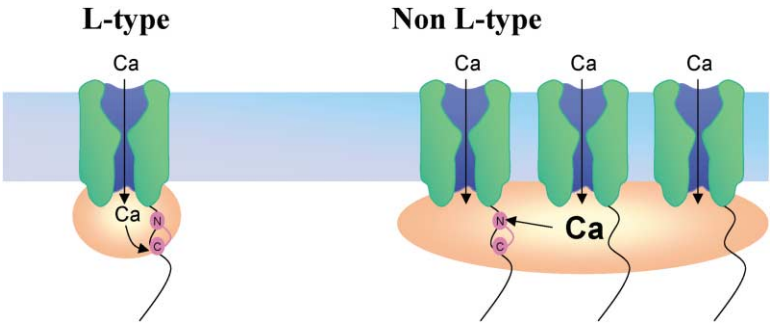

Figure 1. Schematic Representation of Calmodulin Lobe-Specific CDI in L-Type and Non-L-Type Calcium Channels

In non-L-type channels, CDI is mediated by the lower-affinity N-terminal lobe of calmodulin (pink dumbbell), and hence, aggregate increases in intracellular calcium from multiple sources (i.e., channels or internal stores) are required. For L-type channels, in addition to responding to global calcium increases (not shown here), calcium influx through an individual channel is sufficient to trigger CDI due to the high calcium affinity of the C-terminal lobe of calmodulin.

N-type calcium channels allow their overall inactivation profiles to be dominated by either voltage-dependent or calcium-dependent processes.

Although the general presence of CDI appears to be conserved across many calcium channel isoforms, the molecular details differ between L- and non-L-type channels. By overexpressing mutant calmodulin molecules in which calcium binding to either the N-terminal or the C-terminal lobes has been ablated, Liang and colleagues show that, similar to what is seen with P/Q-type channels (de Maria et al., 2001), CDI in N-type and R-type channels is critically dependent on intact low-affinity calcium binding sites in the N-terminal lobe of calmodulin. This contrasts with CDI in L-type channels, which relies predominantly on the high-affinity C-terminal lobe of calmodulin and is consequently insensitive to buffering of intracellular calcium (Peterson et al., 1999). Based on these findings and on calcium diffusion arguments, Liang and coworkers propose that CDI in all Ca_v2 calcium channels occurs primarily in response to global, larger rises in intracellular calcium, whereas the CDI in L-type channels allows these channels to also respond directly to their own passage of calcium ions (Figure 1).

In the presence of high calcium buffer concentrations, the swapping of the C termini of R-type and L-type channels is sufficient to confer CDI properties among these channels (de Leon et al., 1995). This suggests that the use of different calmodulin lobes by these two channels is entirely due to the nature of the C terminus region and that both the R-type and L-type calcium channel backbone contains the necessary machinery that physically inactivates the channel. What these channel structures are and whether CDI and VDI utilize the same molecular pathways remains to be determined. The notion that L-, P/Q-, R-, and N-type channels all share the ability to undergo CDI poses a major challenge toward resolving this issue, as these channels can no longer be considered as suitable templates for further chimeric analysis.

The pronounced CDI in N-type and R-type channels also raises the question as to whether there is any physiological significance to VDI. The authors' finding that VDI becomes enhanced when calmodulin binding to the channel is disrupted suggests the possibility that VDI could serve as a redundant inactivation mechanism in case cellular calmodulin levels were to be downregulated. It is also important to note that steady-state VDI already occurs at voltages at which there is no calcium influx, thus making VDI the key inactivation mechanism that regulates channel availability at physiological resting membrane potentials. In addition, as Liang et al. acknowledge, their study was conducted exclusively with calcium channel complexes containing the β_{2a} subunits, which drastically attenuates VDI. One might thus expect that VDI should predominate over CDI in channels containing, for example, β_3 subunits, which increases VDI. Indeed, the time constants for CDI reported by Liang et al. are about 3- to 4-fold greater than those reported previously for VDI of N-type or R-type channels coexpressed with β_3. Hence, the authors' suggestion that β subunit composition may serve as a molecular switch between VDI and CDI in Ca_v2 channels seems highly appropriate. Finally, it is important to note that T-type calcium channels do not contain IQ motifs or classical EF hand regions in their C termini, suggesting that their inactivation is likely to be exclusively voltage dependent, perhaps because their ultrarapid VDI properties do not necessitate an additional CDI component. Taken together, it appears that both VDI and CDI are essential for regulating calcium entry through voltage-gated calcium channels but that their relative contributions can be adapted to suit a particular cellular requirement.

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Toward a Molecular Description of Pheromone Perception

Successful sexual courtship depends on complex multimodal sensory integration. In this issue of *Neuron*, Bray and Amrein identify a male-specific gustatory receptor gene, *Gr68a*, expressed in leg chemosensory neurons. Genetic ablation of these *Gr68a* neurons suggests that they are intimately involved in perceiving female pheromone cues necessary for mating.

Drosophila fruit fly males, like males of most other species, must display an elaborate series of courtship behaviors in order to persuade females to mate (Figure 1). In a ritual that may seem uncannily familiar to humans, the male begins by locating and orienting toward the female. Next, the male taps the female on the abdomen with his foreleg and follows her if she is in motion. He extends and vibrates his wings in courtship song, then licks her genitalia with his proboscis and bends his abdomen in attempted copulation. The male may pause at any stage in the courtship behavior but will usually resume and persist in this sequence until copulation occurs. The initiation of courtship behavior is dependent upon multiple sensory stimuli. Visual and olfactory cues direct the orientation and following behaviors. Gustatory cues obtained while tapping and licking enable the male to assess the pheromone profile of his potential mate (reviewed in Hall, 1994; Greenspan and Ferveur, 2000). Integration of information from these complex stimuli is necessary for efficient courtship. Which peripheral neurons receive these sex-specific signals and how they are centrally integrated to produce stereotyped behaviors is poorly understood.

Fly courtship behavior appears to be innate, requiring neither previous experience nor learning to be perfected. This poses the intriguing question of how this behavior is genetically programmed. Thus far, investigations into genes influencing courtship behavior have focused on those that control sex differentiation during development. Mutations affecting several transcription factors that act early in the sex determination cascade (*doublesex, dissatisfaction, fruitless*) alter courtship behavior in adult flies. However, the tissue distribution of these genes is rather broad, suggesting a pleotropic effect on many target cells. Further, the downstream targets of these transcription factors are largely unknown, and the extent to which they influence sexual dimorphism in neural organization and wiring is unclear (reviewed in Hall, 1994; Greenspan and Ferveur, 2000). There is much to be learned about how the genetics controlling the organization of each sensory system allows information from external stimuli to be translated into behavior.

In other insect species, pheromones emitted by females are key elements in the process of attracting males into the proximity of receptive females for mating (Hildebrand, 1995). Studies of the neurophysiology and chemical ecology of these long-range moth pheromones have been extremely influential in our understanding of how the nervous system processes odor cues. However, it has not been possible to understand the molecular genetic basis for pheromone perception in these insects. It is therefore of interest to investigate the role of pheromones in *Drosophila*, a genetically tractable species. Cuticular pheromones have been shown to influence the stereotyped behavior exhibited by male *Drosophila* during courtship, acting either to stimulate his serenade toward a potential mate or to inhibit this activity toward other males, recently mated females, or females of other species. The major components of the cuticular pheromone profile are long chain hydrocarbons of limited volatility found predominantly on the abdomen. In *D. melanogaster*, the major female pheromones are 7, 11 heptacosadiene and 7, 11-nonacosadiene, which stimulate male courtship behavior. Males produce 7-tricosene, which inhibits courtship between males (Ferveur and Sureau, 1996). These and other cuticular hydrocarbons are present in varying ratios in different species, promoting courtship among members of the same species and potentially discouraging interspecies mating.

Chemical messages from nonvolatile cuticular pheromones are believed to be detected by contact chemoreceptor neurons located on the male fly, but to date the identity of these neurons has been somewhat mysterious. Males have a greater number of taste bristles on their foretarsi than females (reviewed in Stocker, 1994), and stimuli received through foretarsi have been shown to be sufficient to induce courtship behavior when other sensory organs (antenna, palps, proboscis) are surgically ablated (Robertson, 1983). While observation suggests a role for these organs in pheromone detection during the contact behaviors of courtship, the identity of neurons receiving these chemical signals is poorly understood and the contribution of specific chemosensory receptor proteins has not been previously demonstrated. Several years ago, a large family of gustatory receptor (GRs) genes was identified from genome sequences and found to be expressed on several appendages, including labial palps of the proboscis, foretarsi, maxillary palps, wings, and female genitalia (Clyne et al., 2000; Dunipace et al., 2001; Scott et al., 2001). In this issue of *Neuron*, Bray and Amrein (2003) identify a subset of gustatory receptor neurons (GRNs) that are required for efficient courtship and demonstrate that the gustatory receptor expressed in these cells, *Gr68a*, is responsible for this contribution.

Using *Gr68a*-Gal4 lines to drive the expression of marker proteins β-galactosidase or green fluorescent protein, Bray and Amrein show that *Gr68a* is expressed exclusively in chemosensory neurons innervating taste bristles on male forelegs. Chromosomally female flies with mutations in the sex determination pathway genes